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# Omega-3 polyunsaturated fatty acids alleviate adenine-induced chronic renal failure via regulating ROS production and TGF-β/SMAD pathway

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**Abstract. – OBJECTIVE:** To explore the role of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) in adenine-induced rat chronic renal failure and its underlying mechanism.

**MATERIALS AND METHODS:** 30 Sprague Dawley (SD) rats were randomly assigned into three groups, namely sham group, adenine induction group (adenine group) and adenine induction + ω-3 PUFAs treatment group (ω-3 PUFAs group), with 10 rats in each group. Serum and kidney samples were collected after rats were sacrificed. Serum levels of Cr (creatinine) and BUN (urea nitrogen) were detected using commercial kits. H&E (hematoxylin and eosin) staining was performed to evaluate the pathological changes of kidneys. Levels of oxidative stress indicators in kidney homogenate were detected by commercial kits, including SOD (superoxide dismutase), GSH (reduced glutathione), CAT (catalase), T-AOC (total antioxidant capacity), reactive oxygen species (ROS) production was detected by immunofluorescence. Protein expressions of nuclear factor E2 related factor 2 (Nrf2) and transforming growth factor-beta (TGF-β)/SMAD pathway-related genes were detected by Western blot.

**RESULTS:** Serum levels of Cr and BUN in ω-3 PUFAs group were remarkably decreased compared with those of adenine group. Higher contents of SOD, GSH, CAT and T-AOC were observed in ω-3 PUFAs group compared with those of adenine group. Besides, MAD content and ROS production were lower in ω-3 PUFAs group than those of adenine group. Pathological changes of kidneys were obviously alleviated after ω-3 PUFAs treatment. Western blot demonstrated that ω-3 PUFAs treatment remarkably upregulates Nrf2, HO-1, NQO1, but downregulates relative genes in TGF-β/SMAD pathway.

**CONCLUSIONS:** ω-3 PUFAs alleviated adenine-induced chronic renal failure through enhancing antioxidant stress and inhibiting inflammatory response via regulating Nrf2 and TGF-β/SMAD pathway.

Key Words:
Omega-3 Polyunsaturated Fatty Acids, Nrf2, TGF-β/SMAD pathway, Adenine, Chronic renal failure.

## Introduction

Chronic kidney disease (CKD) is a common disease causing severe economic burden on affected population. The global incidence of CKD is about 10%[1-3]. Early diagnosis and treatment can significantly improve clinical outcomes of CKD. Therefore, precise diagnosis, disease staging and patient management are of great clinical significance[3,4]. In 2002, Kidney Disease Outcomes Quality Initiative (K/DOQI) of National Kidney Foundation (NKF) published guidelines for the assessment and staging of clinical practice for CKD[4]. In this guideline, CKD is used to replace the definition of chronic renal failure (CRF) for better understanding of CKD at different stages. A disease staging system based on glomerular filtration rate levels was also proposed[4,5]. Drug-induced kidney injury is a type of kidney disease caused by exposure to toxins or potentially toxic drugs. The clinical manifestations are abnormal urinalysis, renal pathology, and abnormal renal function[6]. At present, drug-induced nephrotoxicity mainly includes acute kidney injury, chronic kidney disease, acute interstitial nephropathy and nephrotic syndrome[7-9]. Adenine is a commonly used drug with nephrotoxicity, which can lead to CKD. Therefore, prevention and treatment for adenine-induced nephrotoxicity have been well recognized[10]. Secondary damage resulted from CKD should also be urgently prevented, so as to effectively promote CKD treatment[3,5].



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Active oxygen metabolites and inflammatory reactions are considered as important factors leading to drug-induced CKD[8,9]. Reactive oxygen species (ROS) are by-products of biological oxidation reactions, including oxygen ions, peroxides, and oxygen-containing free radicals[10]. Restoration of oxygen supply in damaged tissues leads to great consumption of oxygen by activated phagocytic cells, which is called respiratory bursts[11,12]. Under normal circumstance, ROS production is maintained in a balance *via* a series of reduced substances. However, excessive production of ROS after external stimuli could be overwhelming[13]. Some certain chemical agents such as free radical scavengers, antioxidants and anti-inflammatory cytokines remarkably alleviate tissue damage in renal toxin injury model[14-18]. In addition, transforming growth factor-beta (TGF-β)/SMAD signaling pathway is an essential pathway that controls pro-chronic inflammation and fibrosis gene expressions. It is reported that ROS stimulate tissue damage and inflammatory response *via* activating TGF-β/SMAD pathway[16-18]. In recent years, the impact of Omega-3 Polyunsaturated Fatty Acids (ω-3 PUFAs) on organ damage caused by toxin, ischemia has been well studied[19,20]. In this study, we aimed to investigate the effect of ω-3 PUFAs on adenine-induced CKD and its underlying mechanism. Our findings may provide important evidence for the clinical application of ω-3 PUFAs in adenine-induced chronic renal failure.

## Materials and Methods

### Chemicals and Reagents

ω-3 PUFAs were purchased from Yuanopharm Chemical Reagent (Shanghai, China). Adenine injection was obtained from YiluPharma (Jinan, China). Commercial kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China), including MDA (malondialdehyde), T-AOC (total antioxidant capacity), CAT (catalase), GSH (reduced glutathione), SOD (superoxide dismutase), Cr (creatinine) and BUN (blood urea nitrogen) determination kits. Coulter electronic thermometer and 721-type spectrophotometer were obtained from Ines Analytical Instrument (Shanghai, China).

### Animals and Experimental Protocol

30 adult Sprague Dawley (SD) rats weighing from 180 g were obtained from Vital River Laboratory Animal Technology (Beijing, China). Rats were housed in the environment with a 12 h light/dark cycle and free access to food and water. Rats in sham group were intragastrically administrated with 0.01 ml/g distilled water for 28 consecutive days. Rats in adenine group were intragastrically administrated with 0.01 mL/g distilled water for 28 consecutive days. Meanwhile, intragastric administration of 150 mg/kg·d adenine was performed. The 7th day 2 h after distilled water administration. Rats in ω-3 PUFAs group were intragastrically administrated with 0.01 ml/g ω-3 PUFAs for a total of 28 days. Intragastric administration of 150 mg/kg·d adenine was also performed on the 7th day. Body weight and diuretic activities were recorded during the administration period. This study was approved by the Animal Ethics Committee of Sichuan University Animal Center.

### Assessment of Renal Function

Body weight of rats was daily recorded before intragastrical administration. Bilateral kidney tissues were harvested and weighed immediately after the rats were sacrificed. Kidney index = kidney mass/body mass. 2 mL of blood sample were centrifuged at 3500 g/min for 30 min. Serum levels of Cr and BUN were measured by sarcosine oxidase method and urease method, respectively.

### Histological Examination

Coronal sections of kidney tissues were prepared for histological examination. Kidney sections were fixed with 10% formaldehyde and paraffin-embedded. Tissues were then stained with hematoxylin and eosin (HE) (Boster, Wuhan, China). Histological changes were assessed by semi-quantitative examination of renal tubular necrosis. Evaluation criteria were applied as 0 (no damage), 1 score (<10%), 2 scores (11-25%), 3 scores (26-45%), 4 scores (46-75%) and 5 scores (>76%). Five randomly selected fields of each sample were observed.

### Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) Assay

Apoptosis in kidney sections was detected according to the instructions of *in situ* DNA terminal transferase (TUNEL) assay (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; Chemicon, Millipore, Billerica, MA, USA). Kidney tissues were sliced into 5-μm thick sections and counterstained with methyl green. The number of TUNEL-positive cells in 10 random fields was counted using a high power microscope.

### Biochemical Measurements

Abdominal cavity was exposed by midline abdominal incision. The abdominal aorta was cannulated under the branch of the renal artery, followed by ligation of the proximal segment above the branch of renal artery. The left renal vein was cut open. After the color of kidney tissue changed from red to white, the kidney was quickly removed and placed in liquid nitrogen. Tissues were homogenated for detecting levels of MDA, T-AOC, CAT, GSH and SOD.

For evaluating production of intracellular reactive oxygen species (ROS), intracellular superoxide level assay was detected by a fluorescent microscope (Eclipse Ti-SR, Nikon Co., Tokyo, Japan). The density of the images was detected with a laser scanning confocal microscope (Zeiss Ltd., Göttingen, Germany) in arbitrary units per millimeter square field.

### Western blot

Kidney tissues were added with lysis buffer and shaken on ice for 30 min. The total protein was separated after the centrifugation at 14,000 g/min for 15 min at 4°C. Protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The equal proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures.

### Statistical Analysis

The *t*-test was used for comparisons of continuous variables. Categorical variables were analyzed using $x^2$-test or Fisher's exact probability method. Kaplan-Meier method was performed to evaluate the survival rates of patients and Log-rank test was used to compare the differences between different curves. SPSS 22.0 (Statistical Product and Service Solutions) was used for data analysis (IBM Corp., Armonk, NY, USA). The data were expressed as mean ± standard deviation ($\bar{x}\pm s$). $p<0.05$ was considered statistically significant.

## Results

### ω-3 PUFAs Treatment Improved Renal Function in Adenine-Induced Rats

Body weight and ratio of renal weight/body weight of rats in adenine group were remarkably decreased compared with those of sham group ($p<0.05$), indicating the successful construction of adenine-induced chronic renal failure in rats. Significant improvements of body weight and ratio of renal weight/body weight were found in ω-3 PUFAs group compared with those of adenine group, suggesting that 20 mg/kg ω-3 PUFAs remarkably elevated renal function recovery (Figure 1A and 1B).

Subsequently, we detected serum levels of Cr and BUN in rats of each group. Serum level of Cr was remarkably elevated in adenine group and ω-3 PUFAs group compared with sham group ($p<0.05$). In particular, ω-3 PUFAs treatment could decrease serum level of Cr. However, serum level of Cr in ω-3 PUFAs group was still higher than that of sham group (Figure 1C). Similar results were observed in serum level of BUN (Figure 1D).

### ω-3 PUFAs Preserved Renal Pathologic Structure and Mitigated Neutrophil Infiltration

No significant pathological changes of renal microstructure were found in sham group. Enlarged renal lumen and flat tubular epithelium were observed in adenine group. Besides, disordered cells with granular denaturation and necrosis were shown in renal tissues of adenine group. Significant glomerular contraction, interstitial proliferation and inflammatory cell infiltration were also found. Renal injury in adenine group was less than that of ω-3 PUFAs group (Figure 2A). Similar results were obtained from Masson staining (Figure 2B). Kidney tubules injury score in adenine group and ω-3 PUFAs group was higher than that of sham group ($p<0.05$).

### ω-3 PUFAs Decreased Renal Tubular Cells Apoptosis after Adenine-Induced Renal Injury

We next detected adenine-induced apoptosis in kidney tissues by TUNEL assay. The amount of TUNEL-positive cells in adenine group was remarkably larger than that of sham group. However, ω-3 PUFAs group presented a lower amount of TUNEL-positive cells compared with that of adenine group (Figure 2C and 2D, $p<0.05$).

### ω-3 PUFAs Decreased ROS Production and Tissue Impairment by Enhancing Antioxidant Capacity

It is reported that adenine severely damages antioxidant capacity of kidney and stimulates ROS production. In the present study, we detect-

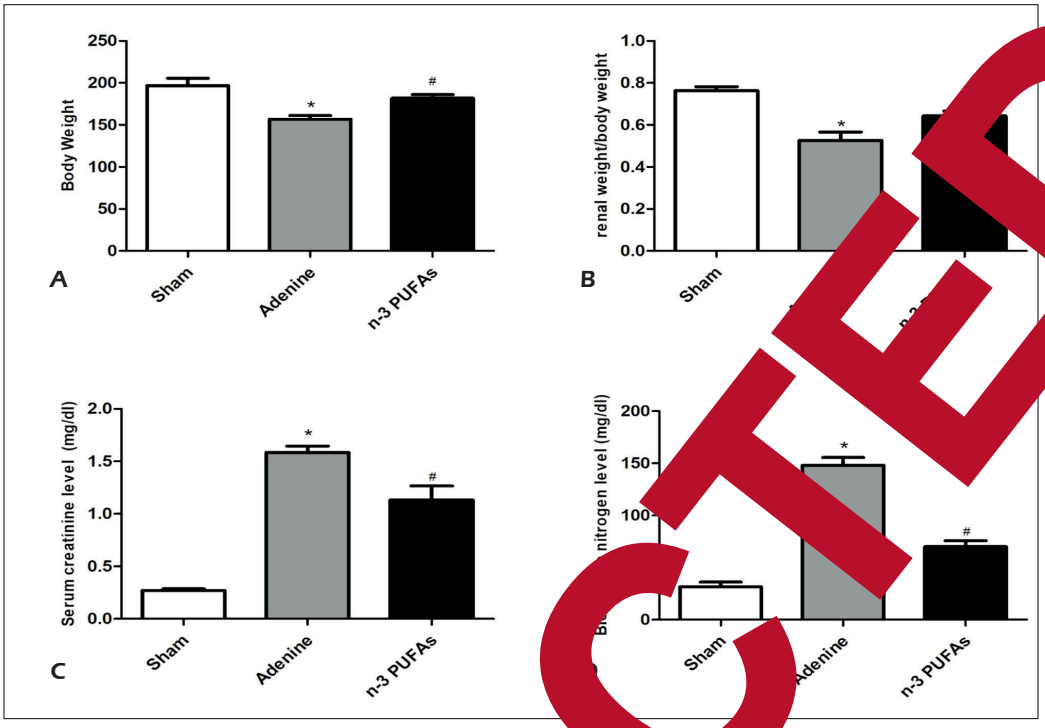




**Figure 1.** 3D model of a sample filled with the Guttacore technique sh… percha *(red)*, cement *(green)*, and voids *(white)*. Figure 1. ω-3 PUFAs conserved renal functi… adenine-induced re… jury. **A**, Body weight of rats in sham group (n=10), adenine group (n=10) and ω-3 PUFAs grou… a ratio of renal weight/body weight in sham group (n=10), adenine group (n=10) and ω-3 PUFAs group (n=10). **C**… in… …ine in sham group (n=10), adenine group (n=10) and ω-3 PUFAs group (n=10). **D**, Serum level of BUN in s… roup (… group (n=10) and ω-3 PUFAs group (n=10). Data were presented as mean±SD, *Significant difference vs… gr… $p<0.05$; #significant difference vs. adenine group ($p<0.05$).

ed antioxidants levels in renal … te using … relative commercial kits. Th… ta de… nstrated that levels of T-AOC, AC… d SOD … e high-er in ω-3 PUFAs grou… thos… group (Figure 3B-3D)… RO… n was also detected using imm… fluores… assay. ω-3 PUFAs pretreatm… emarkably a… ed ROS accumulation (… and 3F). Bes…, MDA level was als… reas… ω-3 PUFAs than that of adenine … roup (Figure …

### *ω-3 PU… Upregulated Nrf2 and … 2 Do… tream Genes by In…ing … Nuc…r Translocation*

To … explor… e mechanism of ω-3 PUFAs … tecti… denine-induced chron-… l fai… collected cytoplasm and nuc… of ad… e-induced kidney tissues, resp… vely. Expression of nuclear Nrf2 was hi… UFAs group than that of sham …p and adenine group (Figure 4A). Western … results also demonstrated stronger nucle-a… location of Nrf2 in ω-3 PUFAs group com… ed with that of sham group and adenine group. Similarly, downstream genes of Nrf2 were also upregulated in ω-3 PUFAs group than those of adenine group, including HO-1 and NQO1 ($p<0.05$). Furthermore, TGF-β/SMAD pathway-related genes were detected by Western blot. The data elucidated that ω-3 PUFAs pretreatment results in downregulated TGF-β, α-SMA, SMAD and FN, as well as upregulated E-cad (Figure 4B), indicating that ω-3 PUFAs regulates adenine-induced chronic renal failure *via* TGF-β/SMAD pathway.

## Discussion

Chronic kidney disease (CKD) is a type of kidney disease in which there is gradual loss of kidney function over a period of months or years[3]. Drug-induced renal failure is a crucial cause of acute kidney diseases. A great number of ROS produced after cardiac macrovascular surgery, kidney transplantation and shock could lead to CKD[2,5]. Studies have shown that Nrf2 is a significant nuclear transcription factor. Nrf2

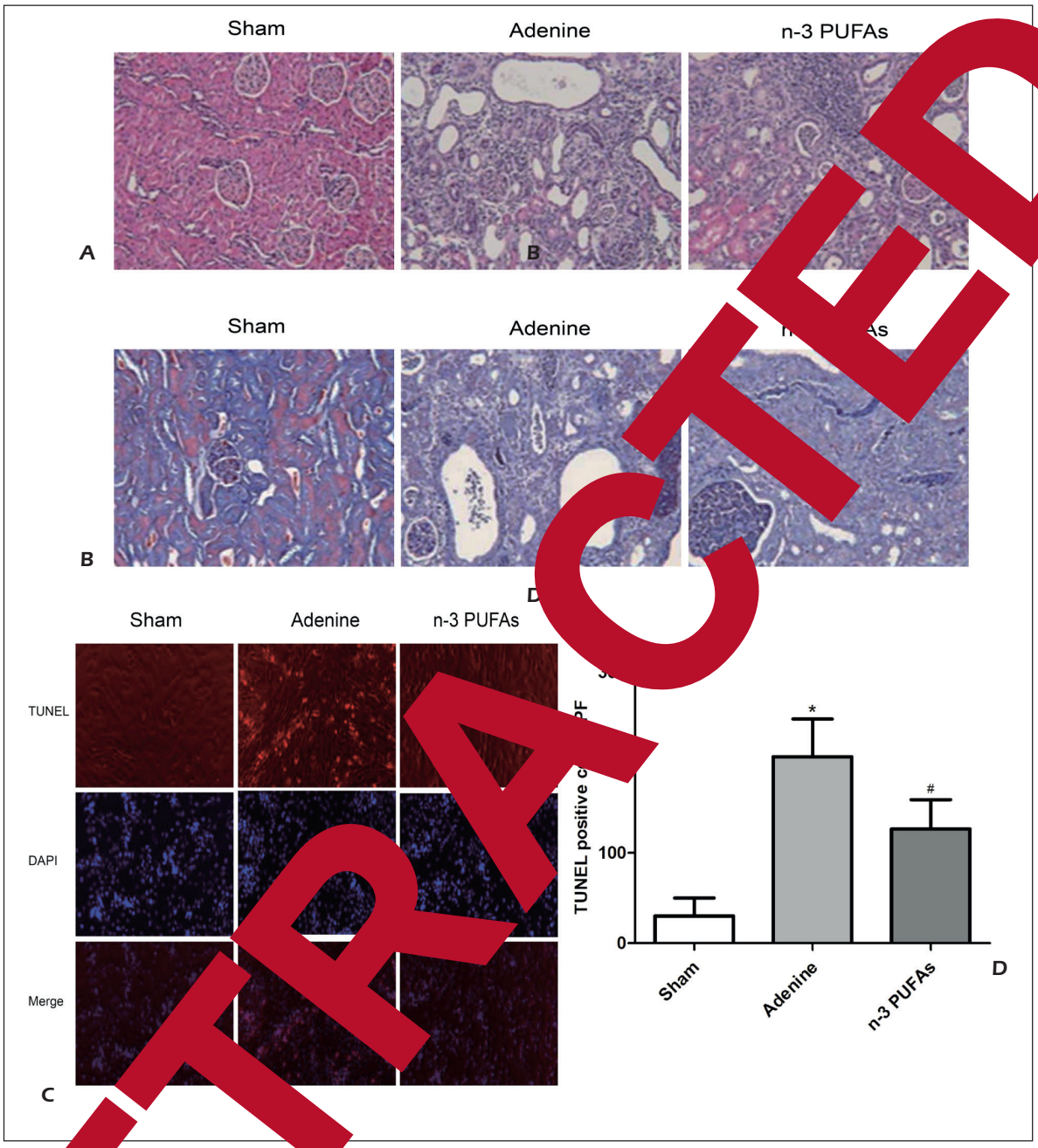


**Figure** 3 PUFA revents adenine-induced renal injury in renal morphology. Renal sections were stained with hematoxylin and e nd exa using a light microscopy (200×). **A**, HE staining of renal tissues in rats of sham group (n=10), adenine gro 0) an PUFAs up (n=10). **B**, Masson staining of renal tissues was assessed the tubulointerstitial fibrosis. **C**, Repres es (mag tion ×100, scale bar=50 μm) of TUNEL immunostaining in the adenine-induced renal injury. **D**, TUNE e cells 3 germ cells of testes. Data were expressed as mean ±SD. *Significant difference vs. sham group 5); #sig di ce vs. adenine group ($p$<0.05).

is c le of defending against oxidative stress. A anti-oxidation response elements E) in the nucleus, Nrf2 regulates expres- levels of multiple downstream antioxidant g [23]. Recent studies have demonstrated that ω-3 FAs is a potent Nrf2 inducer. Functionally, ω-3 PUFAs possess anti-oxidative and anti-apoptotic abilities, which exert a protective effect on drug-induced CKD[19,20]. At present, CKD poses a great burden on the medical resources. In-depth studies are urgently needed to improve the clinical outcomes of CKD[4,5].

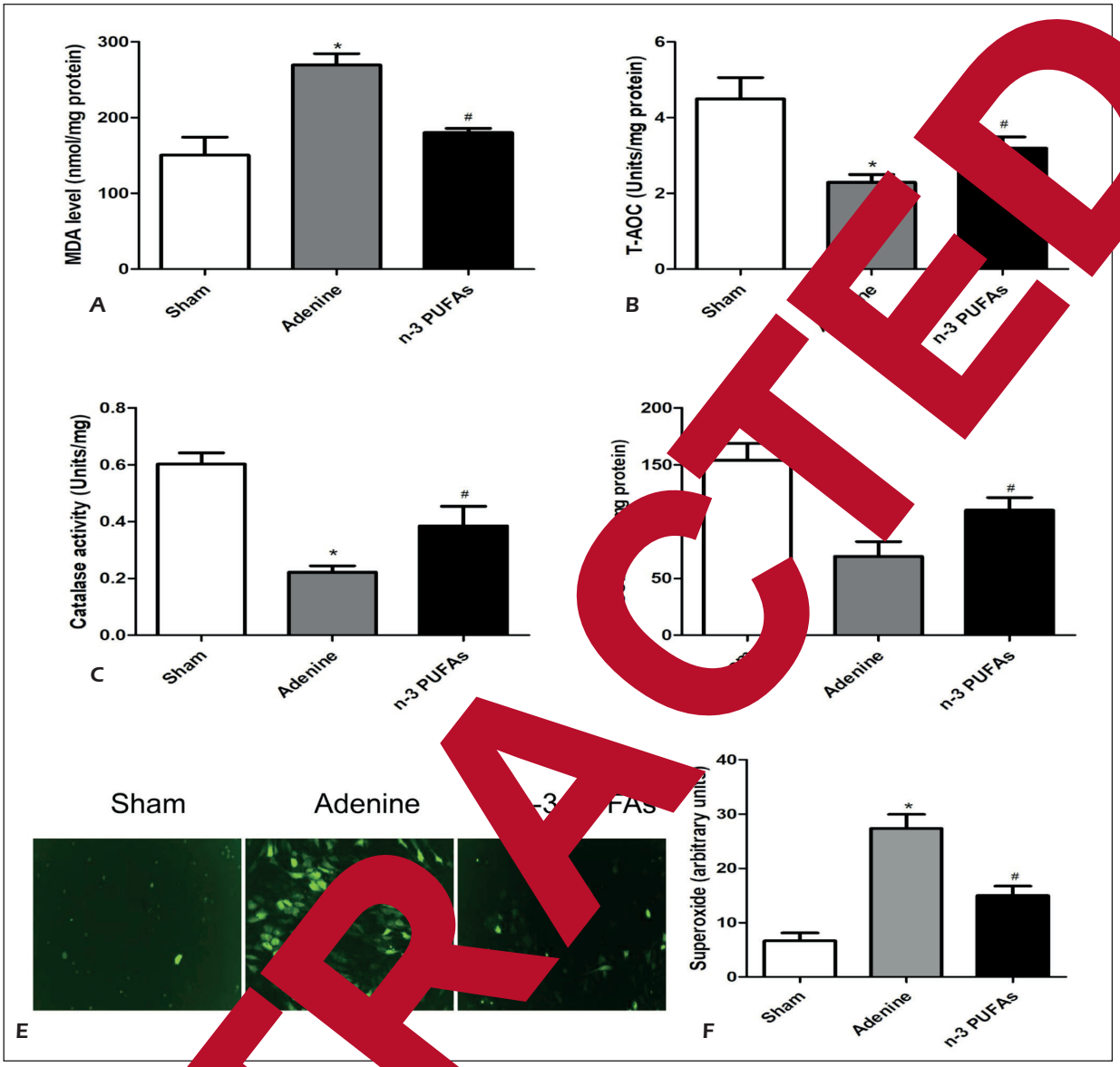


**Figure 3.** ω-3 PU ted oxidative st jury by the assessment of biochemical parameters. **A**, MDA content in kidney tissues. **B**, T co kidney tissues. **C**, CAT content in kidney tissues. **D**, SOD content in kidney tissues. E, DHE staining of kidney sues in s p, adenine group and ω-3 PUFAs group. ROS exhibited red fluorescence under fluorescent microscope. nsity of ROS w ted as arbitrary units per millimeter square field. Data were expressed as mean ±SD. *Significan erence vs. sham grou .05); #significant difference vs. adenine group ($p<0.05$).

tive s is an aptive reaction caused by th ce be n the active oxygen mponen d an dant system. Generally, ve pr ROS exceeds the removal abi of antio t enzymes and antioxidants. Oxi e stress is mainly manifested as inflam- m ltration, increased secretion of eases, and abundance of oxidation interme- as[8-10]. ROS accumulation exerts an important r schemia-reperfusion injury[11]. Hypoxia-induc duction of ATP production and dysfunction of calcium ion channels activate calcium-dependent proteases. Xanthine dehydrogenase is, thereafter, hydrolyzed to xanthine oxidase and accumulated in the lesioned tissues. After oxygen supply is restored in the ischemic tissue, xanthine oxidase is activated to xanthine. Subsequently, superoxide ion radicals are generated and disproportionated to hydrogen peroxide and hydroxyl radicals. The large number of oxygen free radicals damages the function and structure of cells, eventually resulting in cell damage[11-15].

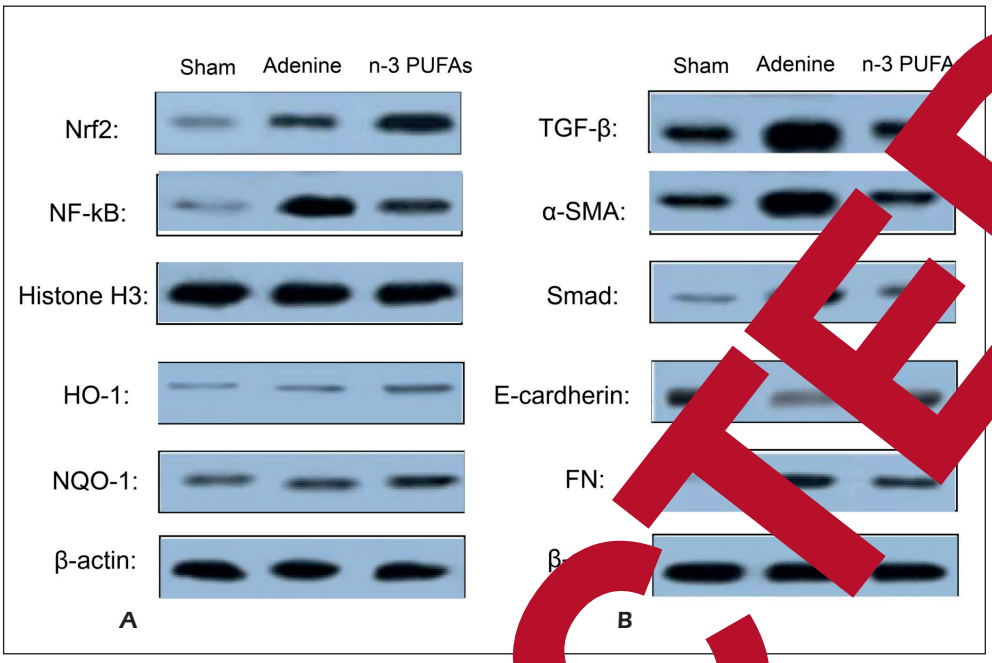


**Figure 4.** ω-3 PUFAs supplementation enhanced Nrf2 nuclear tra ion, increased and NQO-1 protein expression, and decreased TGF-β/SMAD protein expression. **A**, Protein levels of Nr B, HO-1, a QO1 in different groups. Histone H3 was used as a protein control to normalize volume of protein expressio determined by densitometric analysis and normalized to the Histone H3 signal. Protein levels of HO-1 and NQ t groups. β-actin was used as a protein control to normalize volume of protein expression. **B** levels of TGF-β/SMAD in different groups. Data were expressed as mean± SD. *Significant difference vs. sham group t difference vs. ω-3 PUFAs group ($p$<0.05).

Nrf2 is a crucial transcriptional factor volved in oxidative stress. Under normal co ditions, cytoplasmic Nrf2 is d easily degraded[21,22]. However, Nrf disso d from Keap1 and translocated he nucl stimulated by oxidative stre Nucle bind to ARE, a DNA mo g sequence of phase II detoxi ion e genes and antioxidant enzy genes[25]. HO NQO1 reduce oxidati damage by ergistically scaven RO nitrogen species[25,26]. In addition activated also upregulates GSH, GS d SOD, furthe gthening the antioxi function[24].

C ne n k regulation is greatly involved in d induced CKD. Among them, transf owth f -β1 (TGF-β1) is a crucial cyto TGF is secreted by Kupffer which the synthesis of type I proc gen and e III collagen in adjacent hep tellate cells[28]. Scholars[29] have confirmed th induce collagen production in tissue through TGF-β1/SMAD and ERK aling pathways. SMAD protein family is the portant intracellular effector molecule in TGF SMAD pathway. SMAD2 and SMAD3 are phosphorylated by TGF-β1 in renal injury to form hetero-oligomers with SMAD4, thereafter promoting nuclear translocation[30]. SMAD negatively regulates TGF-β1/SMAD signaling pathway *via* inhibiting phosphorylation of SMAD2 and SMAD3[31,32].

ω-3 PUFAs are important components of biological cell membranes. Studies have shown that ω-3 PUFAs exert a variety of physiological functions, such as anti-inflammatory, immune regulation, anti-oxidation, development promotion of the nervous system and retina[19,20]. Animal researches confirmed that ω-3 PUFAs have a protective effect on heart, intestine, liver, brain and other tissues during ischemia-reperfusion injury[19,20,33,34]. However, the effect of ω-3 PUFAs on drug-induced renal failure has not been reported. Our data showed that adenine treatment results in significant histopathological changes, higher levels of oxidative stress and lower antioxidant capacity compared with those of sham group. Levels of MDA and ROS in kidney tissue of ω-3 PUFAs group were decreased, while levels of T-AOC, CAT, GSH, GSH/GSSG and SOD were increased than those of adenine group, indicating that ω-3 PUFAs intervention can reduce oxidative

stress and enhance antioxidant activity. Relative investigations have shown that ω-3 PUFAs can significantly reduce the oxidative stress products and increase the anti-oxidative substances in the lesioned tissues, thereby reducing poison-induced tissue damage[19,20,33]. In this study, Nrf2 was downregulated in adenine group than that of sham group. Besides, expressions of TGF-β/SMAD pathway-related genes were lower in ω-3 PUFAs group than those of adenine group, indicating that ω-3 PUFAs could prevent oxidative stress *via* activating Nrf2 and inhibiting TGF-β/SMAD pathway.

## Conclusions

We found that ω-3 PUFAs alleviated adenine-induced chronic renal failure through enhancing antioxidant stress and inhibiting inflammatory response *via* regulating Nrf2 and TGF-β/SMAD pathway.

### Conflict of Interest

The authors declared no conflict of interest.

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